Cryopreservation of Embryos of the New World Screwworm *Cochliomyia hominivorax* (Diptera: Calliphoridae)

R. A. LEOPOLD,[1] W. B. WANG,[2] D. R. BERKEBILE,[3] AND T. P. FREEMAN[2]

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ABSTRACT A method for cryopreserving embryos of the screwworm, *Cochliomyia hominivorax* (Coquerel), was developed for the long-term storage of strains used in research projects and for colonies maintained as back-up to production strains that are mass-reared for sterile insect release programs. This protocol, when combined with a previous procedure that was designed to render screwworm embryos permeable to water loss and the influx of cryoprotectants, yields rates of ≈53% hatching, 22% pupation, and 75% adult emergence. These yields are all lower than control levels, but by the next generation the progeny of the cryopreserved flies are near or equal to control levels and are available in sufficient numbers to reestablish laboratory colonies. Important to the success of this study was identifying the optimum embryonic stage for treatment, formulating an incubation regime, which allowed consistent retrieval of embryos at the optimum stage, establishing the correct dehydration time for the previtrification step, and developing a recovery system after liquid nitrogen storage of the embryos.

KEY WORDS *Cochliomyia hominivorax*, cryopreservation, screwworm, long-term storage

THE PROPAGATION AND maintenance of back-up insect strains as part of an area-wide, mass-release program or of numerous mutant lines for research projects are often burdensome and costly endeavors. An insect, such as the New World screwworm, *Cochliomyia hominivorax* (Coquerel), which has no intrinsic long-term dormancy capabilities, must be propagated continuously to maintain colonies under laboratory conditions. This activity is subject to the loss of colonies through disease or mechanical failure of rearing facilities and by deliberate elimination during times of budget cuts. Further, undesirable genetic changes can occur within insect colonies through their adaptive responses to laboratory rearing conditions. To alleviate these and other problems associated with insect propagation, storage of insects at some subambient temperature has often been attempted and has resulted in varying success (Leopold 1998).

Ideally, a procedure to be used for preserving insect strains and mutant lines would be economical, allow storage for an indefinite period of time, and cause no change in the genotypic or phenotypic structure of the colony upon reestablishment after storage. Liquid nitrogen has been proven to be the consummate storage medium for preserving a wide array of cells, tissues and organisms. It is easily obtainable, economical, and essentially all life processes are arrested at liquid nitrogen temperature (-196°C). However, most biotic materials cannot be taken directly to liquid nitrogen and must undergo varying amounts of preparation before storage at -196°C and live recovery can be accomplished. This preparation involves reducing the amount of freezable intracellular water to prevent the formation of damaging ice crystals upon lowering the temperature. In addition, to cellular dehydration, the presence of cryoprotectant solutes, such as glycerol or dimethyl sulfoxide, is usually required to protect the cells from injury by the effects of concentrated extracellular solutes and/or physical deformation caused by osmotic shrinkage (Steponkus and Gordon-Kamm 1985, Pegg and Diaper 1988).

Conventional cryopreservation methods involve equilibration of cells or embryos in a permeating cryoprotectant and slow cooling to some sub-zero temperature before quenching and storing in liquid nitrogen. Further, recovery requires warming at some optimum rate before the cryoprotectant is removed. These methods were initially designed for cryopreservation of mammalian embryos but were found to be unuseable for fly embryos, because all the species tested thus far were killed when chilled at temperatures between -10 and -30°C (Heacox et al. 1985, Leibo et al. 1988, Strong-Gunderson and Leopold 1989, Mazur et al. 1992b, Miles and Bale 1995, Leopold and Atkinson 1999). The specific cause for the chilling sensitivity in these dipterans is unknown. However, two similar cryopreservation protocols have been developed for *Drosophila melanogaster* (Meigen) embryos that avoid the damage that is incurred during the sub-zero slow cooling step of the equilibrium freezing technique (Mazur et al. 1992a, Steponkus and Cald-

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[1] Biosciences Research Laboratory, USDA-ARS, Box 5674, Fargo, ND 58105 (e-mail: leopoldr@fargo.ars.usda.gov).

[2] Department of Plant Pathology, North Dakota State University, Fargo, ND 58105.

[3] Midwest Livestock Insects Research Laboratory, USDA-ARS, Lincoln, NE 68583.

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well 1993). These two protocols employ the vitrification procedure of Rall and Fahy (1985) to prepare the embryos so they are able to tolerate storage at liquid nitrogen temperature. This technique involves the rapid cooling of the embryos loaded with highly concentrated cryoprotectant solutions (≈6–8.5 *M*) to effect a glass transition (vitrification) coupled with fast warming to avoid devitrification upon recovery from liquid nitrogen storage.

Considerable modification of the *Drosophila* protocols was required to obtain *Musca domestica* L. adults after cryopreservation as embryos (Wang et al. 2000), whereas only small alterations were made by Nunamaker and Lockwood (2001) to accomplish cryopreservation of embryos of the midge *Culicoides sonorensis* Wirth & Jones. Tailoring a cryopreservation procedure to fit a particular insect may be the rule rather than the exception. We have tested the use of the *Drosophila* procedures of Mazur et al. (1992a) and Steponkus and Caldwell (1993) on five species of dipterans with little success (Leopold and Atkinson 1999, Leopold 2000). Thus, the objective of this study was to develop a new protocol for cryopreservation of the screwworm, *C. hominivorax*, using vitrification methodology. This investigation is a continuation of earlier work involving the development of a permeabilization technique for the screwworm embryo for purposes of gaining the flux of water and permeation of cryoprotectants (Berkebile et al. 2000). The current study describes staging of the embryos, loading with cryoprotectants, the vitrification process, the recovery from liquid nitrogen, and testing of the survival of the cryopreserved insects and their progeny.

Materials and Methods

Collection and Staging of Embryos. Eggs were collected from the New World screwworm strains *Panama 95* (*P95*) and *Costa Rica 92* (*CR92*) as described by Berkebile et al. (2000). The flies were allowed to oviposit for 30 min. Timing for staging of the embryos began at the midpoint of the ovipositional period. After collection, the egg masses were placed into 100-mm petri dishes on two sheets of Whatman grade #1 filter paper (90 mm diameter, Whatman, Hillsboro, OR) that had been moistened with 4 ml of distilled water. The egg masses were then incubated in a nonhumidified chamber using four different temperature regimes: (A) 5 h 45 min at 37°C followed by 30 min at room temperature (RT ≈22°C), (B) 6 h at 37°C and 15 min at RT, (C) 6 h at 37°C and 45 min at RT, (D) 6 h 15 min at 37°C and 30 min at RT.

After incubation, the actual stage of development was also determined visually on embryos that had been dechorionated and permeabilized (see below). The staging was done on embryos that had been placed in a cell culture medium or fetal bovine serum under a dissecting microscope equipped with a transmitted light source in the base of the instrument. Photographs of the embryos were obtained using a Zeiss Axiomat photomicroscope (Thornwood, NY) equipped with a Kodak DC 120 camera and MDS 120 imaging software (Rochester, NY).

Dechorionation and Permeabilization. Removal of the egg shell or chorion with common household bleach and the alkane permeabilization of the vitelline membrane of the embryo was patterned after the method from a previous study (Berkebile et al. 2000). After the hexane extraction of the lipid layer from the vitelline membrane, the embryos were air-dried for 30 s, placed into *Drosophila* Ringer's solution (Chan and Gehring 1971) for 2 min, and gently agitated with a pipette to break up any clumps of embryos.

Cryoprotectant Loading, Dehydration and Vitrification. Two different cryoprotectant loading/dehydration techniques were tested on the permeabilized embryos: (1) after rinsing 2 min in Ringer's solution, the embryos were placed into 1.8 M ethylene glycol (EG) in fetal bovine serum ([FBS], Sigma, St. Louis, MO) for 20 min at ambient temperature to load the embryos with the cryoprotectant. The 20-min time period to fully permeate (load) the cryoprotectant solution into the embryos was determined by visually examining the embryos for an initial shrinkage followed by a reswelling. The embryos were then transferred to a solution of 37.0 wt% EG + 6.0 wt% polyethylene glycol (PEG, MW 6000–7500) + 8.0 wt% trehalose for 8 min on ice to dehydrate the embryos and concentrate the EG; (2) this technique was the same as above except for the dehydration step, where the embryos were placed in 34.0 wt% EG + 5.4% wt% PEG + 15.5 wt% trehalose for 6 min on ice. These two vitrification solutions were used for dehydration in an earlier study and were tested to ascertain whether lowering the concentration of EG from 37 wt% to 34 wt%, increasing trehalose from 8.0 wt% to 15.0 wt%, and decreasing the dehydration time would have a beneficial effect on the hatching rate of the screwworm embryos. For information on the development of these two procedures, refer to Wang et al. (2000).

After the dehydration on ice for both of the procedures, the embryos were transferred to a 25 mm Costar Nucleopore polycarbonate membrane by touching the membrane to the eggs floating on the surface of the solution. The membranes with the adhering embryos were blotted on the reverse side to remove excess solution. Following blotting, the membranes with adhering embryos were placed into nitrogen vapor above a container holding 400–500 ml of liquid nitrogen and held there for 1 min before quickly immersing into the liquid nitrogen. When vitrification occurs, the embryos become a glassy, light yellow in color. Those embryos turning snow white have been killed and indicate that an insufficient amount of cryoprotectant has been loaded into the embryos.

Data Analysis. For purposes of testing the techniques, the embryo samples were held in the liquid nitrogen for time periods ranging from 15 min to overnight. Each replicate throughout this study consisted of two or three membranes having 100–200 embryos attached to each membrane. Thus, if a test was replicated four times, as many as 2,400 embryos may have been processed. Means were analyzed in this study by

using one-way analysis of variance (ANOVA) and the Duncan multiple range test. A difference was considered significant at $P \leq 0.05$.

Warming and Removal of Cryoprotectants. The polycarbonate membranes with the adhering embryos were removed from the liquid nitrogen and again held in the liquid nitrogen vapor for 1 min before being quickly plunged into a solution containing 0.5 M trehalose and 10% FBS in Schneider's insect cell culture medium (SS, Sigma) at ≈22°C. After 2 min, the solution was replaced with FBS for another 2 min, then the membrane and embryos were rinsed three times in FBS at intervals of 10 min. During rinsing, the embryos disengage from the membrane, which was then removed. The embryos were placed into FBS, incubated at 37°C, and the hatching rate determined the following day. The hatching larvae were removed as soon as possible the next day, because growth of microorganisms in the FBS became lethal when larvae were left in this solution longer than ≈18 h. This problem was reduced somewhat by adding the antibiotic, gentimycin sulfate (Sigma), to both the FBS and SS solutions at 10 ppm.

Insect Culture and Colony Reestablishment. The larvae were collected from the FBS and placed on the larval diet and reared to the pupal stage as described by Berkebile et al. (2000). Pupae were collected and weighed individually, allowed to emerge collectively in round paper cartons (4-liter volume) with perforated tops, and the yield of adults was calculated. They were then placed into cages and allowed to mate with each other when mature (5–7 d after emergence). The females were allowed to oviposit their first clutch of eggs at 8–10 d postemergence and the resulting hatch of the F_1 progeny was calculated. These larvae were also cultured to pupation, weighed, and the number of adults emerging was also calculated. Parallel control samples of embryos which had experienced only dechorionation and permeabilization were reared along with samples of cryopreserved embryos and their progeny. Other control samples having no treatment were also examined.

Summary of *C. hominivorax* Embryo Cryopreservation Protocol

Embryo Permeabilization (Berkebile et al. 2000)

1. Break up egg masses by placing in 1.0% NaOH for 5 min and agitating.
2. Rinse eggs with distilled water for 2 min.
3. Agitate in 50% Chlorox bleach for 1 min.
4. Rinse eggs with distilled water for 3 min.
5. Agitate in isopropyl alcohol for 30 s.
6. Air dry for 3 min in a stream of moistened air.
7. Agitate in hexane for 30 s.
8. Air dry for 30 s.
9. Agitate in *Drosophila* Ringer's solution for 2 min.

Loading Embryos with Ethylene Glycol (EG).

1. Place embryos in 1.8 M EG in FBS for 20 min at ambient temperature.

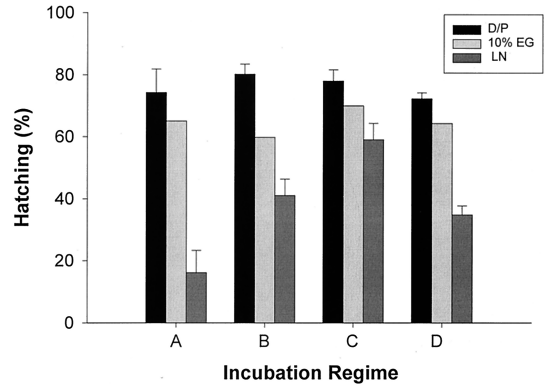

Fig. 1. Egg hatching rates of *C. hominivorax* embryos that had been treated by either dechorionation and permeabilization (D/P); or D/P + loaded with 10% ethylene glycol (10% EG); or D/P + 10% EG + vitrification, stored in liquid nitrogen and recovered from storage (LN). Each treatment was submitted to one of four preincubation regimes. (A) 5.75 h at 37°C + 30 min at ≈22°C. (B) 6.0 h at 37°C + 15 min at 22°C (C) 6 h at 37°C + 45 min at ≈22°C (D) 6.25 h at 37°C + 30 min at ≈22°C. Vertical error bars indicate ± SEM ($n = 4$ for D/P and LN; $n = 2$ for 10% EG; 200–600 embryos per replicate).

Dehydration in Vitrification Solution.

1. Place embryos in 37 wt % EG + 6 wt % PEG (polyethylene glycol) + 8 wt % trehalose for 8 min on ice.

Vitrification and Storage in Liquid Nitrogen.

1. Transfer embryos to polycarbonate membrane.
2. Blot reverse side of membrane to remove excess vitrification solution.
3. Hold membrane with embryos in liquid nitrogen vapor for 1 min.
4. Plunge membrane with embryos into liquid nitrogen.
5. Transfer membranes with vitrified embryos to suitable containers for cataloging while in liquid nitrogen storage.

Recovery from Liquid Nitrogen.

1. Remove membrane with adhering embryos from liquid nitrogen storage.
2. Hold membrane above liquid nitrogen in vapor for 1 min.
3. Quickly plunge membrane into solution containing 0.5 M trehalose and 10% FBS in SS (fetal bovine serum and Schneider's cell culture medium) and hold for 2 min at ambient temperature.
4. Replace solution with FBS and hold for 2 min.
5. Wash membrane and embryos three times with FBS at intervals of 10 min and remove membrane.
6. Leave embryos in FBS overnight for hatching and then place larvae on larval diet.

Results

Figure 1 shows the survival of screwworm embryos to hatching after treatments of dechorionation/per-

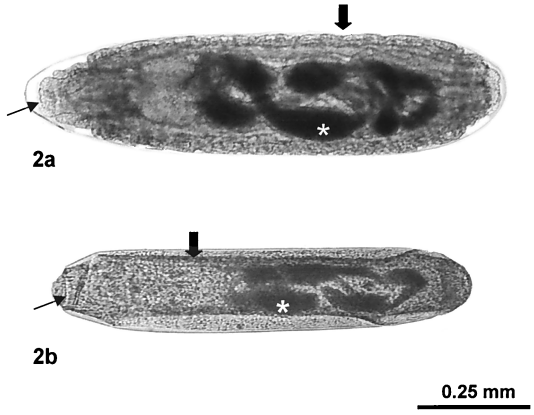

Fig. 2. Upper photomicrograph (2a) shows the stage of *C. hominivorax* embryonic development which is optimum for beginning cryopreservation treatment. It has been dechorionated and permeabilized. The small arrow depicts head involution, the larger arrow shows the extent of external segmentation, and the asterisk shows the coiling of the midgut containing the darkened remnants of yolk. The lower micrograph (2b) shows a shrunken embryo of *C. hominivorax*, which has been dechorionated, permeabilized, loaded with EG and dehydrated in the vitrification solution for 8 min. The small arrow shows wrinkling in the head region. The larger arrow marks one of the longitudinal folds and the asterisk the lightened, coiled midgut.

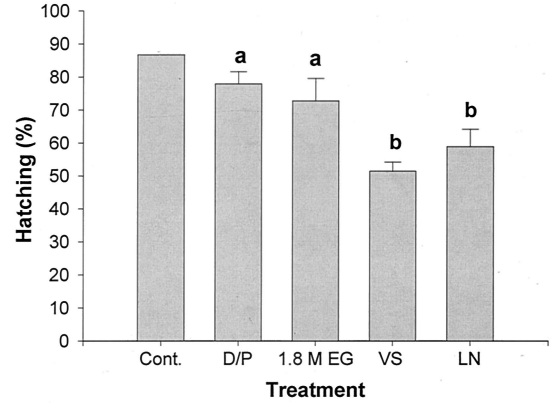

Fig. 3. Hatching rates of *C. hominivorax* embryos that had been either left untreated (Cont., control); dechorionated and permeabilized (D/P); D/P + loaded with 1.8 M ethylene glycol (1.8 M EG); D/P + 1.8 M EG + dehydrated in vitrification solution on ice (VS); or D/P + 1.8 M EG + VS, then vitrified, stored in liquid nitrogen and recovered from storage (LN). All treatments were preincubated with the postoviposition regime of 6 h at 37°C + 45 min at ≈22°C. (n = 2 for Cont., all others n = 4; 200–600 embryos per replicate) Vertical error bars indicate ± SEM Columns with different letters at top are significantly different at $P < 0.05$ (Duncan multiple range test).

meabilization, cryoprotectant loading with ethylene glycol, and dehydration/vitrification at four different postoviposition incubation regimes. The pretreatment incubation regime C (6 h at 37°C followed by 45 min at ≈22°C) clearly elicited the best survival to hatching after dehydration/vitrification and recovery of the embryos ($P < 0.001$). With the other two steps of the protocol that were tested in this study, dechorionation/permeabilization and EG loading, the differences in survival between the four regimes were inconclusive. Because the untreated control egg hatching for the two strains used in this study typically ranges from 85 to 90%, it is evident that each successive treatment step of each incubation regime tested has toxic effects. However, the best survival for the entire cryopreservation protocol was obtained when embryos were held for 6 h at 37°C followed by 45 min at ≈22°C before treatment.

Visual examination of the embryos showed that most were in the developmental stage as shown in Fig. 2a when they had experienced the pretreatment incubation regime C. The gut displays several darkened coils containing the last remnants of yolk and head involution and segmentation is evident. Regimes A and B typically produced embryos with little coiling of the gut and the formation of the head is indistinct (not shown). Embryos given pretreatment incubation regime D often displayed muscular movements and may have air in their tracheae (not shown). Also, these embryos undergo little shrinkage when placed into the 1.8 M EG, which indicates that the development of the embryonic cuticle has occurred and permeation of cryoprotectant has been blocked.

Figure 3 shows a comparison of egg hatching rates for embryos treated with one to four of the successive steps of our protocol, dechorionation/permeabilization, loading with 1.8 M EG dehydration in vitrification solution, and vitrification followed by recovery from liquid nitrogen storage. Preceding each succession of treatments, the incubation time of 6 h at 37°C and 45 min at ≈22°C was used, and following all treatments the standard recovery procedure was implemented. The control egg hatching rate was nearly 87%, and we determined that the dehydration and vitrification/recovery steps reduces the survival significantly from the dechorionation/permeabilization and EG loading steps ($P < 0.05$). Although the dehydration step did not differ from the vitrification/recovery in this test ($P > 0.05$), it consistently yielded lower survival when embryos were left in the in the vitrification solution too long. In this case, the embryos that were dehydrated without going through the vitrification step were processed after the parallel samples had been vitrified. This means that these embryos spent more time in the vitrification solution than did those experiencing vitrification before recovery. Thus, the cooling and warming procedure appears to be less damaging to the embryos than an increased period of dehydration in the vitrification solution.

There was no significant difference between hatching rates of the *P95* and *CR92* strains following treatment with the optimum incubation period and the vitrification protocol ($P = 0.39, n = 11$). The *P95* strain had hatching of 58.0 ± 3.2 and *CR92* a rate of 52.4 ± 5.2. Because these numbers were not significantly different, the mean hatching rate on Table 1 represents

Table 1. Yield of *C. hominivorax* embryos after cryopreservation and during the subsequent F_1 generation

Treatment	Highest hatching (%)	Egg hatching ± % SE ($n = 11$)	Pupation ± % SE ($n = 4$)	Pupal wt (mg) ± % SE ($n = 4$)	Adult emergence ± SE ($n = 4$)	♂/♀ Ratio ($n = 4$)
LN	63.9	$52.5^a \pm 2.1$	$21.9^a \pm 7.2$	$35.5^a \pm 2.7$	$74.0^a \pm 5.9$	1.03^a
PERM	83.2	$76.2^b \pm 1.7$	$53.3^b \pm 8.6$	$36.2^a \pm 1.6$	$87.3^b \pm 5.7$	1.02^a
CONT	85.0	$73.9^b \pm 9.3$	$70.8^b \pm 12.5$	$42.1^b \pm 3.3$	$82.5^{ab} \pm 3.9$	0.96^a
F_1 Generation						
LN	—	78.2	—	$47.0^b \pm 3.9$	$94.7^c \pm 1.7$	1.04^a
PERM	—	75.5	—	$43.6^b \pm 1.8$	$96.7^c \pm 0.3$	1.01^a
CONT	—	86.6	—	$44.2^b \pm 5.4$	$93.3^c \pm 3.6$	1.02^a

LN = liquid nitrogen cryopreservation, PERM = permeabilization control, CONT = untreated control. $n = 200–600$ embryos. Numbers within columns with different letters are significantly different at $P < 0.05$ (Duncan multiple range test).

n = 4 for the mean egg hatching of the parental control groups.

n = 2 for the mean control egg hatching of all F_1 treatment samples.

the combined hatching data from both strains. All other data, except that given in the next paragraph, are from treatment of the *P95* strain.

Hatching of embryos dehydrated for 8 min in the solution having more EG and less trehalose was 52.4 ± 5.2%, and hatching for embryos treated with the lesser amount of EG greater trehalose and 6 min dehydration was 40.1 ± 7.5% ($n = 4$). Although the latter solution appeared to yield a mean hatching rate that was 12% less than the solution with the higher EG concentration and lower trehalose, the two rates were not different statistically because of the wide variation of the replicates ($P = 0.22$). However, we used the solution having the greater EG less trehalose, and longer dehydration in the development of our protocol because the handling of the samples was easier when using the longer dehydration time.

Dehydration of embryos in the concentrated EG/PEG/trehalose vitrification solution (6.8 M) produces notable morphological changes. Fig. 2b shows an embryo that was incubated in the solution for 8 min on ice and depicts the stage of development and dehydrated appearance that is typical of embryos before a successful vitrification. The anterior end is wrinkled and there is a fold running on each lateral side of the embryo that compresses and distorts the coiled form of the gut. Exterior evidence of segmentation is not visible, and the overall shading of the dehydrated embryos is lighter than untreated embryos. This lighter appearance is caused by severe flattening of each embryo in addition to the wrinkling and folding that occurs upon loss of cellular fluids.

Table 1 compares egg hatching, pupation, and adult emergence data of cryopreserved embryos with embryos that had experienced only the dechorionation and permeabilization steps of the protocol and with a control group that had no treatment. Following cryopreservation, there were significant reductions in the rates of hatching ($P < 0.001$), adult emergence, and pupation ($P < 0.05$), whereas the mean weight at pupation and the male/female ratio of the cryopreserved groups were similar to the permeabilized control. The greatest reduction in survival occurred during the larval stages, where only 22% of the hatched larvae pupated. When calculated as a percentage of the untreated controls, the total yield of adults after using this protocol was ≈12%. It was also noted that even though the pupal weights of the cryopreserved and permeabilized groups did not differ ($P = 0.83$), they were significantly less than those of the untreated control and their F_1 progeny ($P < 0.05$).

The data in the lower half of Table 1 shows the egg hatching, pupal weight, and adult emergence of the progeny obtained from the parental treatment groups. Except for a higher hatching rate of eggs from the untreated group ($P < 0.05$), there were no differences in hatching of the other two groups and no differences between all three groups with respect to pupal weights, adult emergence and male/female ratio ($P \geq 0.05$). Thus, by the following generation, the progeny of the cryopreserved flies have essentially regained the measured characteristics of the untreated groups and we obtained hundreds of insects with which to reestablish the colony.

Discussion

As pointed out by Mazur et al. (1992a), the stage of dipteran embryonic development most tolerant to the physical and chemical stress elicited by the process of cryopreservation is far more differentiated than most other nonanhydrobiotic organisms that have been successfully preserved at the temperature of liquid nitrogen (-196°C). For example, mouse embryos survive best when frozen at eight cells (Wittingham et al. 1972) and larvae of the nematode, *Caenorhabditis elegans*, at 558 cells (Sulston et al. 1983). In comparison, the *Drosophila* embryo at 12–13 h postoviposition has ≈50,000 cells (Anderson and Lengyel 1981) and it is the 13.5- to 14.5-h-old embryo that is best able survive cryopreservation (Steponkus and Caldwell 1993). Thus, as we show with the screwworm embryo in Fig. 2a, the status of embryonic development, when compared with other studies of dipteran developmental rate versus level of differentiation (Cantwell 1976, Campos-Ortega and Hartenstein 1985), is ≈75–80% completed when we begin our treatment procedure. There is no conspicuous evidence to explain why this stage of embryonic development is more tolerant to the cryopreservation procedure than the other stages.

It was suggested by Leopold (1991) that the abundance of yolk and/or the occurrence of numerous cell divisions may cause the extreme sensitivity to chilling which has been observed for the young embryos (Heacox et al. 1985, Leibo et al.1988, Strong-Gunderson and Leopold 1989, Mazur et al. 1992b, Miles and Bale 1995, Leopold and Atkinson 1999). In comparison, organogenesis is well on the way to completion and there is little yolk remaining during the stage of development that was used for cryopreservation in this study. Further, it is the last stage that can be permeated with cryoprotectants because there is an external, impermeable cuticle produced on the surface of the embryos shortly after we recover them from liquid nitrogen storage.

In addition to the permeabilization procedure that we have reported previously (Berkebile et al. 2000), there are other steps in our cryopreservation protocol for *C. hominivorax* where correct timing is critical to the successful completion of the process. As mentioned earlier, obtaining the stage of embryogenesis most tolerant to the procedure is one of these critical steps. At an incubation temperature of 37°C, the time period from oviposition to hatching is only ≈8.5–9 h for this insect, and we found that the time span to obtain and treat the correct stage within the time to complete embryogenesis is relatively small (≈15 min). To cope with this small window of opportunity, we lengthened the end of the incubation period by removing the embryos from the 37°C chamber and placed them at room temperature. This had the effect of slowing down embryonic development and gave us the opportunity to visually assess the stage of development and to make minor adjustments in the 45-min pretreatment period, if needed. Thus, our incubation period was structured by carefully balancing two different holding temperatures to obtain the correct treatment stage.

Mazur et al. (1992a) and Steponkus and Caldwell (1993) have each used different methods in their *D. melanogaster* cryopreservation protocols for obtaining the optimum stage for treatment. Steponkus and Caldwell (1993) used the chronological age of 13.5–14.5 h at 25°C as the determinant of embryo readiness for treatment, and they maintained that the visual staging of embryos was less precise and subjective. Mazur et al. (1992a) used visual staging of embryos after holding 20 h at 17.5°C, which yielded an ≈1:1 ratio between the stages 14 and 15, as defined by Wieshaus and Nüsslein-Volhard (1986). They asserted that visually defining the optimum developmental stage would allow for any strain-to-strain differences in developmental rate and small variations in incubation temperature, which could produce cryobiologically significant differences in tolerance to their cryopreservation procedure. We recently found that visual staging of embryos after an incubation period to be essential for certain dipterans, e.g., some of the species of tephritid fruit flies, which require up to 5 d before hatching. These insects commonly display variations in embryonic developmental rates, even though oviposition and incubations time remained constant (R.A.L. and W.B.W., unpublished data).

Another feature of our protocol, where strict attention to timing must be given, is the dehydration step right before vitrification is initiated. This dehydration step concentrates the cryoprotectant within embryos to a level where it is possible to vitrify the embryos when cooled at a sufficiently rapid rate (Rall and Fahy 1985). As illustrated in Fig. 2b, the embryos are severely shrunken after 8 min in the hyper-osmotic vitrification solution. However, our previous studies indicate that a variation of just 2 min of exposure to the vitrification solution can determine whether hatching will or will not occur (Wang et al. 2000). Whether this response is caused by toxicity elicited by the vitrification solution directly or dehydration damage due to concentration of endogenous cellular solutes is not currently known. However, Mazur et al. (1993) concluded from their experiments on concentrating ethylene glycol within *D. melanogaster* embryos with nonpenetrating solutes that the rise in intra-embryonic concentration of ethylene glycol was more damaging than dehydration.

The current protocol that we have developed for cryopreservation of *C. hominivorax* embryos can be used for long-term storage of colonies maintained in the laboratory and also for those back-up strains used in the mass production of flies for autocidal control via sterile fly release programs. Although the uncorrected yield of adults derived from the cryopreserved embryos is ≈9%, the yield was sufficient to reestablish colonies from different strains. Testing of additional strains of *C. hominivorax* which have inherent low viabilities when reared in the laboratory will determine how serviceable this method is for widespread use. Rall et al. (2000) determined that female genotype was an important factor to be considered when establishing banks for human disease models using rat and mouse embryos. The genotype of donor females affected the number of embryos collected and subsequently recovered after cryopreservation. Similar studies will also be required to determine whether sufficient numbers of survivors are being recovered to maintain desired gene and genotype frequencies of insect strains when using our cryopreservation protocol.

It is expected that continued use of our protocol will bring improvements that will increase the overall yield of adults. For example, Steponkus et al. (1990) initially reported a 3% yield of adults coming from cryopreserved *D. melanogaster* embryos which hatched at rate of ≈18%. After optimization of their protocol, this low yield was increased to an overall rate of ≈45% adult emergence from cryopreserved embryos hatching at rate of ≈83% (Steponkus and Caldwell 1993). Further, Mazur et al. (1992a) reported that ≈25% of cryopreserved *Drosophila* embryos survived to the adult stage with their protocol, a 50-fold increase over their earlier efforts (Mazur et al. 1993).

With both of the *Drosophila* cryopreservation protocols, the greatest increase in survival resulted when the most tolerant stage of development was identified and used for treatment by vitrification. While identifying the most tolerant stage of development for our

procedure was an important part of this study, reducing the toxicity of the vitrification solution, and/or the dehydration phase, appears to be the key to increasing survival after cryopreservation for *C. hominvorax* embryos and other insects we have studied (Leopold and Atkinson 1999, Leopold 2000, Wang et al. 2000).

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